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# *Acanthamoeba* spp.: *In vitro* effects of clinical isolates on murine macrophages, osteosarcoma and HeLa cells

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ABSTRACT

Three different cell lines (murine macrophages, HeLa and osteosarcoma cells) were assayed in order to check for the manifestation of the cytopathic effects of three strains of *Acanthamoeba* recently isolated in our laboratory from contact lens cases: CLC-16, CLC-41.r and CLC-51-l. Adhesion and cytotoxicity assays were carried out with these strains and the type strain *Acanthamoeba castellanii* Neff as a control. Briefly, the ability of these amoebae to bind to the three cell lines was calculated and supernatants were examined for cytotoxicity by measuring lactate dehydrogenase released as an estimate of cytotoxicity using a commercial detection kit. The three strains showed high adhesion and cytotoxicity levels when tested in the three cell lines. This study demonstrates the ability of these amoebae to degrade any of the tested cell lines. To the best of our knowledge, this is the first report of the *in vitro* effects of acanthamoebae on osteosarcoma cells.



## 1. Introduction

*Acanthamoeba* species are opportunistic free-living amoebae that are causative agents of various human pathologies such as a type of fatal encephalitis called *Acanthamoeba* Granulomatous Encephalitis (AGE) and disseminated infections in immunocompromised patients and Amoebic Keratitis (AK) in immunocompetent patients, mainly contact lens wearers (Marciano-Cabral and Cabral, 2003; Schuster and Visvesvara, 2004).

So far, there are not effective available treatments for the pathologies caused by *Acanthamoeba*. Moreover, due to the rare occurrence of these infections and the lack of specific clinical symptoms, there is often a delay in the diagnosis that could be fatal. Other aspects to take into account are the low penetration levels in the Central Nervous System (CNS) of most of the antiparasitic drugs and also the ability of *Acanthamoeba* to form cysts, stage that allows them to resist the action of any external agent (Khunkitti et al., 1998; Marciano-Cabral and Cabral, 2003; Schuster and Visvesvara, 2004; Khan, 2006).

*Acanthamoeba* pathogenesis is highly complex and involves several steps. Overall, the ability of the amoebas to bind to host cells is the first crucial step in the pathogenesis of *Acanthamoeba* infections (Cao et al., 1998; Kennett et al., 1999; Mattana et al., 2009). This event triggers the activation of secondary events, such as secretion of toxins, interference with host intracellular signalling pathways and phagocytosis of host cells. Finally causing host cell death, that can occur by necrosis or apoptosis (Alizadeh et al., 1994; Khan, 2006; Mattana et al., 2009).

Evolutionary studies have led to the identification of 15 different genotypes (T1–T15) based on rRNA gene sequencing (Stothard et al., 1998; Horn et al., 1999; Gast, 2001; Booton et al., 2005). The correlation between pathogenicity and certain genotypes continues to be investigated (Stothard et al., 1998; Booton et al., 2005; Maghsood et al., 2005). To date, studies have shown that 90% of *Acanthamoeba* isolates that produce infections belong to the T4 genotype, suggesting that the abundance of T4 isolates in human infections is most likely due to their greater virulence and/or properties that enhance their transmissibility, a higher prevalence in the environment as well as their decreased susceptibility to chemotherapeutic agents (Maghsood et al., 2005; Khan, 2006). Moreover, pathogenic *Acanthamoeba* strains are able to produce and secrete different enzymes (mainly proteases) in order to damage the host cell/tissue and allow amoebic invasion of the infected cell/tissue (Lorenzo-Morales et al., 2005; Clarke and Niederkorn, 2006; Khan, 2006). Thus, the identification of potentially pathogenic strains is based on proteases zymograms and other biochemical assays such as the ability to grow at high temperatures and/or

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osmotic pressure conditions (Lorenzo-Morales et al., 2005; Khan, 2006). Previous studies using different human cell lines, have demonstrated that these events are both contact-dependent and contact-independent mechanisms of cellular damage (Marciano-Cabral and Toney, 1998; Alsam et al., 2003; Khan, 2003, 2006; Sissons et al., 2004, 2005).

In this study, the *in vitro* effects of three potentially pathogenic clinical isolates of *Acanthamoeba* on three eukaryotic cell lines (murine macrophages, HeLa and osteosarcoma) were evaluated using adhesion and cytotoxicity assays. Furthermore and to our knowledge, this is the first report of *in vitro* effects of acanthamoebae on osteosarcoma cells.

## 2. Material and methods

### 2.1. Acanthamoeba strains

Three clinical isolates (CLC-16, genotype T3; CLC-41.r, genotype T4 and CLC-51, genotype T1) obtained in a previous study in our laboratory (Martín-Navarro et al., 2008) and the type strain *Acanthamoeba castellanii* Neff (ATCC 30010, genotype T4) were used in this study. Previous use in the assays, the four strains were axenically grown in PYG medium [0.75% (w/v) proteose peptone, 0.75% (w/v) yeast extract and 1.5% (w/v) glucose] containing 40 μg gentamicin $ml^{-1}$ (Biochrom AG, Cultek, Granollers, Barcelona, Spain).

### 2.2. Eukaryotic cell lines

The cell lines that were used in this study: J774A.1 murine macrophages (ATCC # TIB-67), HeLa cells (ATCC # CCL-2) and the U-2 OS osteosarcoma cell line (ATCC # HTB-96) were purchased from the American Type Culture Collection (ATTC, LG Promochem, Barcelona, Spain). These cells lines were chosen based on their role in the host's system: macrophages were selected as there are related to the host's immunological response. Moreover, J774A.1 cells are active in antibody-dependent phagocytosis, synthesize large amounts of lysozyme and exhibits minor cytolysis but predominantly antibody-dependent phagocytosis. Finally, osteosarcoma are bone-related cells and there are not studies, to our knowledge reporting the ability of *Acanthamoeba* to bind and/or degrade these cells. The HeLa cell line was used as a control for the study.

The three cell lines were maintained as monolayer cultures at 37 °C in 5% $CO_2$ in T-75 culture flasks (Corning, Madrid, Spain). Cells were routinely cultured in Dulbecco's Modified Eagle Medium without phenol red (DMEM, Sigma, Tres Cantos, Madrid, Spain) supplemented with 10% foetal calf serum, 2 mM glutamine and 20 μg $ml^{-1}$ gentamicin (Biochrom AG, Cultek, Granollers, Barcelona, Spain) and subcultured two times per week.

### 2.3. Adhesion assays

To determine the ability of the *Acanthamoeba* strains used in this study to bind to the three assayed cell lines, adhesion assays were performed as previously described (Alsam et al., 2003; Sissons et al., 2004). Briefly, cell lines were grown to a confluent monolayer in 6-well plates, 24 h prior to the assay. Amoebae strains ($10^5$ cells/well) were added to the plates and incubated at 37 °C in 5% $CO_2$. After 1 h of incubation, the unbound amoebae were removed by washing with 1× Phosphate Buffer Saline (PBS) and counted using a Z1 series COULTER COUNTER® cell and particle counter (Beckman Coulter, Madrid, Spain). The percentage of bound amoebae was calculated as 100%–% unbound amoebae, being the % unbound amoeba, the total of added amoebae minus the number of unbound amoebae.

### 2.4. Cytotoxicity assays

Cytotoxicity assays were performed using a cytotoxicity detection kit based on the release of lactate dehydrogenase enzyme (LDH) (Roche Diagnostics, Sant Cugat del Vallès, Barcelona, Spain) as previously described (Alsam et al., 2003; Lorenzo-Morales et al., 2005). Briefly, the three cell lines were grown to monolayers in 6-well plates. *Acanthamoeba* strains ($10^5$ amoebae/well) were incubated with cell monolayers in foetal calf serum free DMEM medium at 37 °C in 5% $CO_2$. Cell monolayers were observed periodically for cytopathic effects for up to 24 h. At the end of this incubation period, cytopathic effects were assessed visually after haematoxylin staining. In addition, supernatants were collected and cytotoxicity was determined by measuring LDH release following manufacturer's instructions. Control values were obtained from each cell line incubated alone in foetal calf serum free DMEM without phenol red. Total LDH release was determined from each cell line treated with 2% Triton X-100 (Sigma, Tres Cantos, Madrid, Spain).

## 3. Results and discussion

The tested clinical strains were able to adhere to the three cell lines in a high percentage between 93% and 99% (Table 1). Moreover, cytotoxicity assays revealed that the three clinical isolates of *Acanthamoeba* were producing severe damage on the three cell lines within 10–12 h of incubation as determined visually by haematoxylin staining. At 24 h, cytopathic effects were more extensive and resulted in the complete loss of cell monolayers in the three cell lines (Fig. 1D–F).

To further determine whether these cytopathic effects are due to the lysis of the cell lines or simple disruption of the cell monolayers, LDH release was determined in the supernatant. Results showed high percentages of cytotoxicity at 24 h (Table 1).

Moreover, cytotoxicity assays revealed that the three clinical isolates were able to kill between 54.44% and 84.61% of the cell monolayers (Table 1, Fig. 1). When cells incubated with amoebae were examined by microscopy, amoebae were observed to be able to ingest any of the cell line including the osteosarcoma cell line (Fig. 1C and F).

In our study, adhesion and cytotoxicity assays were performed simultaneously with three clinical isolates recently isolated in our laboratory and belonging to genotypes T1, T3 and T4. All the strains were able to adhere and degrade cell monolayers of three different cell lines: murine macrophages, HeLa and osteosarcoma cells. No major differences in the adhesion and cytotoxicity rates were ob-

**Table 1**
Percentages of adhesion and cytotoxicity (LDH release) at 24 h after inoculation of the three clinical isolates of *Acanthamoeba* and the type strain *A. castellanii* ($10^5$ cells/well) used in this study with the eukaryotic cell lines.

| | HeLa[a] | Macrophages[b] | U-2 OS[c] |
|---|---|---|---|
| *% Adhesion* | | | |
| CLC-16 | 95.8 | 93.2 | 97.5 |
| CLC-41.r | 95.8 | 94.6 | 97 |
| CLC-51.l | 97.1 | 97.3 | 94.8 |
| AcNeff[d] | 97.5 | 98.2 | 99 |
| *% Cytotoxicity* | | | |
| CLC-16 | 74.64 | 54.44 | 75.58 |
| CLC-41.r | 66.25 | 56.6 | 84.61 |
| CLC-51.l | 71.78 | 61.1 | 77.51 |
| AcNeff | 70.41 | 63.3 | 68.02 |

[a] HeLa cells (ATCC # CCL-2).
[b] J774A.1 murine macrophages (ATCC # TIB-67).
[c] U-2 OS osteosarcoma cell line (ATCC # HTB-96).
[d] *Acanthamoeba castellanii* Neff (ATCC 30010).